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13. ABSTRACT (Maximum 200 Words)

Dysregulation of the human Transforming acidic coiled coil (TACC) genes is thought to be important in the development of breast cancer. However, the mechanism by which they function still remains to be clarified. We have demonstrated that the full length TACC2 protein can inhibit the tumorigenic phenotype of certain breast cancer cells. Similarly, the TACC2 interacting protein, hGCN5 can also reduces breast cancer cell survival. The catalytic domain of this histone acetyltransferase is critically important for this effect. Thus, modulation of hGCN5 and/or pCAF activity may represent a mechanism by which TACC2 may exert its tumor suppressive properties. We have furthered our analysis of TACC2 mediated transcriptional events by examining the effects of TACC2 on the BRCA1 mediated regulation of the p21 gene. Our initial data suggests that basal levels of p21 and STAT1 are decreased in TACC2 transfected cell lines. Independently, we have found an interaction between the TACCs and the FHL family of transcriptional regulators. These latter proteins coactivate or corepress transcription, in a promoter specific context. We are now examining whether TACC2 possesses similar properties, or alternatively acts as a transcriptional switch, as previously indicated for the CBF-1/RBP-Jκ splice variant of FHL1 in the Notch signaling pathway.

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INTRODUCTION

The human transforming acidic coiled-coil (TACC) family of genes map to chromosomal regions associated with the development and progression of cancer (1,2). TACC2 is normally expressed at low levels in normal breast cells (3). Recently, Chen et al demonstrated that TACC2 mRNA was downregulated in the more malignant clones of the HMT-3522 cell line based model for breast tumor progression (3). In our first year report, we described the reevaluation of the potential role of TACC2 as a breast tumor suppressor gene, based on our identification of the two major isoforms of TACC2 expressed during development, including the splice variants expressed in the mammary gland. The analysis of TACC2 overexpressing cell lines revealed cell type specific effects on the ability of breast cancer cells to exhibit anchorage independent growth and to migrate through a basement membrane like matrix, suggests that the effect of overexpression of TACC2 on the ability of breast cancer cells to divide in culture could be differentially affected by the genetic background of the original tumor, and may link the function of TACC2 to estrogen signaling.

There is significant evidence to indicate that TACC2, and TACC proteins, in general, are distributed both in the cytoplasm and the nucleus (4), with some of the nuclear localised TACC protein concentrated in nuclear speckles (3). This indicates that both the TACC proteins and histone acetyltransferases can be physically located in the same subcellular compartment. We have previously determined that TACC2 interacts with the histone acetyltransferase hGCN5 (5). As hGCN5 is a key component of complexes that regulate transcription by acetylating histones and transcription factors (6,7), this suggested that TACC2 could play a role in the regulation of transcription through interaction with this molecule. We demonstrated that, in the human embryonic kidney cell line 293, the native TACC2s isoform co-immunoprecipitates with the histone acetyltransferases hGCN5, pCAF, CBP and p300. As outlined below, we are further characterizing the normal functional role of TACC2, with particular relevance to these interactions with the histone acetyltransferases, and a potential functional role in transcriptional regulation in response to interferon-γ (IFN-γ) stimulation. This analysis is already providing insights into the role of TACC2 in the normal growth and differentiation of cells, and possible mechanisms by which inactivation could promote tumor development.

This is the third year report for this grant covering the twelve month period from July 1/2003 – June 30/2004. At the time of the submission of the previous annual report, the work outlined was under review for publication in Oncogene. We were requested to perform some additional experiments, which were not originally a part of this grant proposal, but were absolutely required prior to acceptance of the manuscript. The manuscript was finally published in Oncogene, Volume 23 (see Appendix)

BODY

Specific Aim 2: Examination of the role of hGCN5 and the TACC2-hGCN5 interaction in breast tumorigenesis.

Task 5: Construction of hGCN5 deletion mutants and determine their effect on breast cancer cell lines

We have previously reported that hGCN5 was expressed at relatively low levels in breast cancer cell lines, although, the closely related gene, pCAF is expressed in the same lines and may therefore represent an alternative target for potential TACC2 mediated transcriptional events. During the reporting period, we have performed a detailed analysis of the role of hGCN5 in breast cancer cells. Due to the low transfection efficiency, and the inability to identify transfectants stably expressing hGCN5 or its

deletion constructs, we have resorted to the use of a retroviral system. Thus, we have cloned hGCN5 and a series of deletion constructs spanning the HAT domain, and/or the ADA2 interaction domain and the C-terminal bromodomain into the retroviral vector pLPLNCX. In each case, the hGCN5 construct was fused to green flurorescent protein, in order to readily confirm infection efficiency. We have used these pLPLNCX-hGCN5 retroviruses to infect the several breast cancer cell lines, including MCF10, MCF7 and MDA-MB-468, and selected for integration of the retroviral construct with neomycin. In all cases, excluding the retrovirus containung the N-terminal region of hGCN5 (DeltaHAT-ter), relatively few clones survived. Furthermore, we detected relatively little expression of the hGCN5 constructs in those stable clones produced, suggesting selection against hGCN5 expressing clones. In combination with the previous observation that lack of expression of hGCN5 in breast cancer cells, this data further indicates that hGCN5 may be a breast tumor suppressor gene in its own right. This completes Task 5 in the Statement of Work.

Specific Aim 3. Characterization of the effect of TACC2 on the transcriptional enhancement of BRCA1 by CBP/p300.

Task 7: Detection of TACC2 and hGCN5 in the CBP/p300/BRCA1 complex

We have demonstrated that TACC2 is found in a complex with CBP/p300 in the HEK293 cell line. Thus, we have proposed that TACC2 and hGCN5 may be present in the complex of CBP/p300 and BRCA1. Downregulation of TACC2 or hGCN5 in breast cancer may therefore represent an alternative mechanism of inactivation of BRCA1 function, comparable with that seen in hereditary breast cancer. We have performed a series of immunoprecipitations to determine the presence or absence of TACC2 in the BRCA1-CBP/p300 complex in breast cancer cells (Task 7). In these cases, to avoid potential artifacts caused by overexpression of the target proteins, we have attempted to immunoprecipitate the native TACC2 and BRCA1 proteins. To date, this has failed to demonstrate a direct interaction in breast cancer cells between these proteins. However, recent data from large scale protein interaction studies in *C. elegans* has confirmed a direct functional interaction of the *C. elegans* TACC protein with the *C. elegans* homologue of the BRCA interacting protein BARD-1 (8). Furthermore, we have recently identified a functional interaction between TACC2 and FHL2 (four and half LIM domain protein 2), which is also known to interact with BRCA1 (9). Thus it remains possible, that TACC2 either indirectly interacts with BRCA1 via BARD1 or FHL2, or competes with BRCA1 for complex formation with one or both of these proteins.

Task 8/9: BRCA1 plasmid construction, determination of the role of TACC2 in the BRCA1 mediated regulation of the p21 promoter

In our original proposal, we intended to examine the effect of TACC2 on GAL4-BRCA1 mediated induction of a GAL4 responsive expression cassette containing the chloramphenicol acetyl transferase gene. As BRCA1 has now been shown to enhance the IFN-γ mediated induction of the cyclin dependent kinase p21WAF1 (10), we have sought to investigate the direct effect of TACC2 on this BRCA1 regulated promoter. Thus, we have isolated and cloned the full length and the C-terminus of BRCA1 into the mammalian expression vector pcDNA3. Similarly, we have cloned the p21 promoter into the pSEAP reporter vector (BD Bioscience Clontech). Activation of the p21 promoter-SEAP reporter should result in the transcription, synthesis and subsequent secretion of SEAP (secreted alkaline phosphatase) into the tissue culture media of responsive cell lines. The SEAP activity is then determined

using a luminometer to measure the light produced by the conversion of the chemiluminescent substrate CSPD by the active SEAP enzyme in the media and the kinetics of induction of the response element can be followed over time by measuring the accumulation of SEAP in the culture media. Transfection efficiencies can be determined by cotransfection of a plasmid that constitutively expresses the DsRed fluorescent protein, and either counting fluorescent cells, manually or using a fluorimeter, at each time point that the SEAP activity is measured. Alternatively, the cells can be cotransfected with a β-galactosidase expression plasmid, and enzyme activity measure at the end point of the experiment for normalization. Thus, after normalization, the effect of TACC2 on transcriptional activation from the p21 reporter can be determined by measuring the difference in the accumulation of SEAP in the culture medium of cell lines transfected with the full length TACC2 relative to control cells transfected with vector alone.

We have already used this system to determine the affect of the TACC proteins on the transcriptional regulation of the cyclic-AMP response element (*unpublished data*). In turn, in initial studies of the p21 reporter, we have determined that TACC2 reduces the basal level of this promoter in HEK293 cells. Furthermore, p21 levels are reduced in breast cancer cell lines stably expressing TACC2 from a CMV promoter (Fig 1). In an Affymetrix gene chip experiment (funded by the Roswell Park Alliance Foundation), we have independently determined that TACC2 downregulates the basal transcription level of the STAT1α gene. As this protein is required for IFN-γ mediated induction of p21, we performed a Western blot to determine whether p21 downregulation correlates with reduced STAT1α protein. This does indeed occur (Fig 1) indicating that IFN-γ signaling could be compromised in these cells. Currently, we are further investigating the effect of TACC2 on the expression of other components of the BRCA1/STAT1α complex. Another possibility is that, similar to CBF-1/RBP-Jκ (FHL1) transcription factor in Notch signaling (11), TACC2 may act as a promoter specific transcriptional switch, changing from a transcriptional repressor to an activator, depending upon pathway activation. This point will be clarified by our studies on the induction of p21 by IFN-γ in the presence of stably and transiently upregulated TACC2 (Task 9).
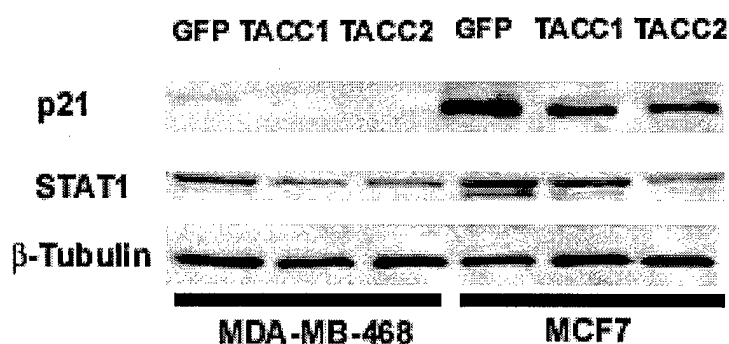

Fig 1. Western blot analysis of p21 and STAT1 protein expression in breast cancer cell lines transfected with TACC1 or TACC2 expression plasmids. This indicates that basal expression of p21 is reduced in TACC overexpressing cell lines. This appears in part to be due to a reduction in the basal level of STAT1.

Thus this completes Task 8 (cloning of BRCA1 expression plasmids), leaving Task 9 (transcriptional analysis of IFN-γ signaling) to be fully completed. Due to the late recruitment of Dr. Gangisetty in 2001 (as detailed in the first year report), we have received approval for a no cost extension to this project to complete this last Task by the end of November 2004.

SUMMARY STATUS OF TASKS OUTLINED IN THE STATEMENT OF WORK
Task 1   Complete
Task 2   Complete

Task 3   Complete
Task 4   Complete
Task 5   Complete
Task 6   Complete
Task 7   Complete
Task 8   Complete
Task 9   In progress

KEY RESEARCH ACCOMPLISHMENTS

1) Inhibition of proliferation of breast cancer cells by hGCN5 is mediated by the HAT and ADA domain.
2) BRCA1 does not directly bind to TACC2, but may bind or compete with BRCA1 in a ternary complex with FHL2 and/or BARD1
3) TACC2 lowers the basal expression of p21, and the STAT1α gene in MCF7 and MDA-MB-468

REPORTABLE OUTCOMES

1)   Development of retroviral constructs expressing subdomains of hGCN5
2)   Construction of p21 report plasmids

Publications
1)   Gangisetty O, Lauffart B, Sondarva, G, Chelsea D, and **Still IH**. (2004). The Transforming acidic coiled coil proteins interact with nuclear histone acetyltransferases. Oncogene 23:14:2559-2563.

CONCLUSIONS
Through the course of this proposal, we have analyzed the effect of the novel tumor suppressor gene, TACC2 and one of its binding partner, hGCN5, on the cellular dynamics of breast cancer cell growth. We have determined that the expression of full length hGCN5 as well as constructs containing the histone acetyltransferase catalytic site are detrimental to breast cancer proliferation. In combination with the previous observation that breast cancer cells do not express significant levels of hGCN5, this data further indicates that hGCN5 may be a breast tumor suppressor gene in its own right. Our previous studies have suggested that TACC proteins may perform an assembly or coordination function bringing elements of the chromatin remodeling, transcriptional and posttranscriptional machinery together in the nucleus. In part this may also be due to the TACC2 protein in these complexes counteracting a negative modulator of histone acetylation activity. Our data from this current year's work, suggests that TACC2 can reduce the basal level of p21, in part through downregulation of STAT1. This would appear to counter our previous hypothesis that TACC2 is an activator of growth inhibitory pathways in the normal breast epithelium. However, the lack of detectable binding of TACC2 to BRCA1 could suggest that overexpression of TACC2 competes with BRCA1 for the transcriptional coregulator, FHL2. Another possibility, is that, as noted for the CBF-1/RBP-Jκ (FHL1) transcription factor in Notch signaling (11), TACC2 may act as a promoter specific transcriptional switch, changing from a transcriptional repressor to an activator, depending upon cell signaling pathway activation. Further analysis of the effect of TACC2 on the induction of p21 by IFN-γ should resolve this issue, and clarify the role of TACC2 in IFN-γ signaling in breast cancer.

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APPENDIX

Reprint of
Gangisetty O, Lauffart B, Sondarva, G, Chelsea D, and **Still IH**. (2004). The transforming acidic coiled coil proteins interact with nuclear histone acetyltransferases. Oncogene 23:14:2559-2563

The transforming acidic coiled coil proteins interact with nuclear histone acetyltransferases

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Dysregulation of the human transforming acidic coiled coil (TACC) genes is thought to be important in the development of multiple myeloma, breast and gastric cancer. However, even though these proteins have been implicated in the control of cell growth and differentiation, the mechanism by which they function still remains to be clarified. Using the yeast two-hybrid assay, we have now identified the histone acetyltransferase (HAT) hGCN5L2 as a TACC2-binding protein. GST pull-down analysis subsequently confirmed that all human TACC family members can bind in vitro to hGCN5L2. The authenticity of these interactions was validated by coimmunoprecipitation assays within the human embryonic kidney cell line HEK293, which identified the TACC2s isoform as a component consistently bound to several different members of HAT family. This raises the possibility that aberrant expression of one or more TACC proteins may affect gene regulation through their interaction with components of chromatin remodeling complexes, thus contributing to tumorigenesis.

Recently, we described the cloning and genomic structure of the transforming acidic coiled coil 2 gene (TACC2) (Lauffart et al., 2003), a member of the evolutionarily conserved TACC family of genes (Still et al., 1999a). These genes encode proteins that are highly acidic and contain a conserved 200 amino-acid coiled coil domain, termed 'the TACC domain', which we previously predicted to play a critical role in the function of these proteins (Still et al., 1999a). Both in vitro and in vivo studies indicate that the TACC proteins are functionally linked to the processes of cell growth and differentiation (Gergely et al., 2000; Lauffart et al., 2003; Sadek et al., 2003). In fact, functional deletion of the Tacc3 gene in mice is embryonically lethal, with

homozygous knockout mice dying during mid to late gestation (Piekorz et al., 2002). At present, however, evidence indicates a contrasting role for the TACC family in processes underlying the development of cancer. On the one hand, the expression of specific TACC1 isoforms has been implicated in the development of gastric cancer (Line et al., 2002). On the other, the TACC1 protein has been shown to be significantly reduced in 50% of resected breast tumors, compared to normal levels, suggesting that TACC1 may be a breast tumor suppressor gene (Conte et al., 2002, 2003). TACC2 has also been suggested to be a candidate tumor suppressor gene (Chen et al., 2000; Conte et al., 2003; Lauffart et al., 2003). Overexpression of the short isoform, TACC2s, can reduce the in vitro tumorigenic properties of estrogen receptor negative breast cancer cell lines (Lauffart et al., 2003); an effect that is apparently mediated by the conserved TACC domain (Chen et al., 2000; Still et al., unpublished).

It is known that all the TACC proteins identified to date interact via the TACC domain with the microtubule-binding proteins of the stu2/msps/ch-tog family, and the Aurora Kinases, and that these interactions are required for the accumulation of ce-TAC-1, D-TACC and the vertebrate TACC3 proteins to the centrosome (Lee et al., 2001; Giet et al., 2002; Le Bot et al., 2003; Srayko et al., 2003; Tien et al., 2003). However, although initially described as centrosomal proteins (Gergely et al., 2000; Gergely, 2002), during interphase the human TACC proteins are actually distributed throughout the cell, with TACC1 and TACC3 being primarily concentrated in the nucleus (Gergely et al., 2000). Nuclear accumulation of TACC3 has also been noted during normal mouse development (Aitola et al., 2003; Sadek et al., 2003). Thus, the distribution and behavior of the TACC proteins resembles those of signaling intermediates that shuttle between the cytoplasm and the nucleus, suggesting that TACC proteins may have additional functions outside their potential role at the centrosome and during mitosis. Indeed, TACC2 has been shown to migrate to the nucleus upon stimulation of microvascular endothelial cells with erythropoietin, implicating TACC2 in erythropoietin signaling (Pu et al., 2001). Furthermore, we have recently demonstrated that human TACC1 and TACC2 interact with the potential nuclear oncogene, glioma

amplified sequence 41 (GAS41) (Lauffart et al., 2002, 2003) and that TACC2s is found in a complex with the INI-1 core component of the SWI/SNF complex, presumably through its interaction with GAS41 (Lauffart et al., 2003). Mouse Tacc3 can coactivate the transcriptional response to hypoxia, by binding to the arylhydrocarbon nuclear translocator (ARNT1) transcription factor (Sadek et al., 2000), directly implicating a TACC family member in transcriptional control. We now present further evidence for an intimate role for the TACC proteins in transcription by describing their physical association with the histone acetyltransferase (HAT) family of transcriptional coactivators. This raises the possibility that aberrant expression of one or more TACC proteins may affect gene regulation through their interaction with components of chromatin remodeling complexes, thus contributing to tumorigenesis.

To further clarify the potential function of the TACC proteins in the interphase cell, we have initiated a yeast two-hybrid based screen to identify proteins that interact with the conserved TACC domain of TACC2s. We fused the TACC2s open reading frame from amino acid (aa) 767–1094 to the GAL4 DNA-binding domain of the pAS2.1 vector. This construct was transformed into CG1945, and lack of autoactivation and lack of nonspecific interactions confirmed between the bait TACC2 protein and the GAL4 activation domain and human lamininC negative control proteins. The TACC2 bait was then used to screen a fetal brain cDNA library (BD Biosciences Clontech), as previously described (Lauffart et al., 2002). Approximately 10^6 transformants were plated and selected on His$^-$ selective media containing 10 mM 3-aminotriazole. Of the 30 His$^+$ clones initially isolated, six proved positive when assayed for β-galactosidase activity using the colony lift assay. The numbers of clones isolated during this procedure were comparable to other published manuscripts, including those on TACC proteins (Conte et al., 2002; Lauffart et al., 2002), suggesting that these clones represented bona fide TACC2 interacting partners. Subsequent isolation and sequence analysis revealed that one of these clones, FB6, corresponded to the carboxy terminal 215 aa's of the HAT hGCN5L2 (Figure 1a). This region contains the bromodomain (BrD), which has been identified in several transcriptional regulatory proteins, and the ADA2 interaction region (Xu et al., 1998) (Figure 1a). To confirm that the interaction between hGCN5L2 and TACC2s is direct, and to further refine the binding site of TACC2s on hGCN5L2, we next assessed this novel interaction using the glutathione-S-transferase (GST) pull-down assay. Deletion and smaller fragments of hGCN5L2 were cloned in pET28c and tested for their ability to interact with TACC2s. TACC2s was expressed in E. coli as a GST fusion protein, incubated with each in vitro translated hGCN5L2 subfragment, and subjected to several wash steps to remove any nonspecific binding. As can be seen in Figure 1b, TACC2s was found to bind specifically to the ADA2 interaction region, but was unable to interact with the BrD, when this region was expressed separately. Thus, the interaction of TACC2
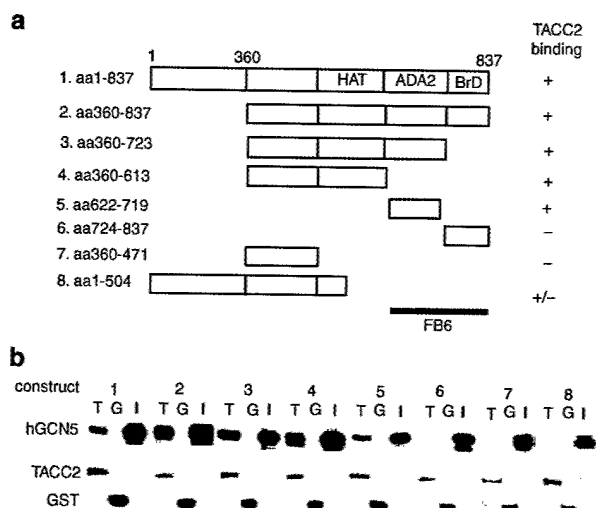
Figure 1 In vitro interactions between TACC2s and hGCN5L2. (a) Diagram and summary of deletion constructs for hGCN5L2-binding domain mapping. Extents of the constructs are defined by the reference protein sequence NP_066564. The original clone identified by yeast two-hybrid (FB6) included the ADA2 interaction domain and the BrD. (b) Interaction of TACC2 with hGCN5L2 constructs by GST pull-down. Lane numbering corresponds to the numbered hGCN5L2 construct in (a). Top panel: Autoradiograph of 12% SDS polyacrylamide gels with in vitro translated hGCN5L2 construct pulled down with GST-TACC2s (T) or GST (G). I: 5% input of hGCN5L2 construct; bottom two panels represent Coomassie blue stained gels of the pull-down experiment, verifying equal loading of the GST fusion proteins. Full-length TACC2s was cloned into pGEX5X2 (Amersham Biosciences, Piscataway, NJ, USA), and GST-fusion protein prepared and immobilized on glutathione sepharose beads, as previously described (Lauffart et al., 2003). Subclones of hGCN5L2 were generated by PCR (primers sequences available on request) and cloned into pET28c (Novagen, Madison WI, USA). These constructs were used to synthesize ^{35}S-radiolabeled proteins by the TNT quick coupled transcription/translation system kit (Promega, Madison WI, USA), according to the manufacturer's instructions. In total, 5 µg of immobilized GST protein was incubated with 60% of the in vitro translated protein in HEPES binding buffer (20 mM HEPES, pH 7.5, 100 mM NaCl, 12% glycerol and protease inhibitors) at 4°C for 90 min. Unbound labeled protein was removed by washing four times with binding buffer. Bound proteins were then eluted by boiling in GST elution buffer (100 mM Tris-HCl, pH 8.0, 20 mM reduced glutathione) and analysed on 12% SDS polyacrylamide gels. Dried gels were autoradiographed

with the yeast two-hybrid clone FB6 was due to the specific binding of the coiled coil domain of TACC2 to the ADA2 interacting domain contained within this clone. In addition, a second TACC2-binding domain in hGCN5L2 was also detected: TACC2s interacted with those constructs that contained the intact HAT domain, and bound weakly to construct 8, which contained the N-terminal section of the HAT domain. Lack of binding to aa 360–471 further suggested that TACC2 specifically binds to the ADA2 interaction domain and the intact HAT catalytic domain of hGCN5L2.

The TACC family of proteins is defined by a highly conserved C-terminal 200 aa coiled coil domain (TACC domain) (Still et al., 1999a, b). The TACC domain of TACC2 shows highest homology to that of TACC1

(74% identity) (Still et al., 1999a). Thus, the finding that this region of TACC2 binds to hGCN5L2 suggested that the other TACC family members could also bind to this HAT. To address this issue, TACC1 (aa596-ter) and TACC3 (aa116-ter) were fused to GST and assayed for binding to in vitro synthesized hGCN5L2, as described above. Figure 2 shows that both these proteins bind directly to the hGCN5L2 protein, confirming that the TACC proteins, as a whole, are a family of hGCN5L2-binding proteins.

Given that the TACC2s-binding site on hGCN5L2 is highly related to pCAF (79% identity), it would seem likely that TACC2s could also interact with pCAF. Furthermore, previously, Yang et al. (1996) demonstrated that pCAF and hGCN5L2 form complexes with two other highly related HATs, that is, the CREB-binding protein (CBP) and p300, and different combinatorial interactions between these proteins can govern the transcriptional activation of a number of promoters by specific transactivators. Thus, to further examine whether native TACC2s can be found complexed with other HATs in vivo, we next performed coimmunoprecipitation experiments with commercial antibodies individually raised against native hGCN5L2, pCAF, p300 and CBP. Figure 3a demonstrates that in the human embryonic kidney cell line 293, native TACC2s coimmunoprecipitates with all these four proteins. We next examined whether a highly specific commercial antibody raised to an internal region of human TACC2s (Lauffart et al., 2003), which does not overlap the proposed HAT-binding site, could immunoprecipitate the native TACC2–pCAF complex from HEK293 and the breast cancer cell line MCF7. In both cell lines, this antibody immunoprecipitates a TACC2 complex that contained endogenous pCAF (Figure 3b), further strengthening the conclusion that TACC2s binds to pCAF in human cells.

We have previously demonstrated that overexpression of TACC2s can reduce the in vitro tumorigenic properties of some breast cancer cell lines (Lauffart et al., 2003), a process that appears to be mediated by the conserved TACC domain (Chen et al., 2000; Still et al., unpublished). Furthermore, the involvement of HATs in human cancer has now been demonstrated (Borrow et al., 1996; Ida et al., 1997; Sobulo et al., 1997; Chaffanet et al., 1999; Gayther et al., 2000), and the hGCN5L2 gene has been suggested to be a candidate tumor suppressor in those cases of ovarian and breast cancer that show loss of heterozygosity in chromosome 17q21, without BRCA1 mutations (Tangir et al., 1996; Niederacher et al., 1997). Therefore, we were particularly interested in determining whether the TACC proteins could interact with hGCN5L2 and pCAF
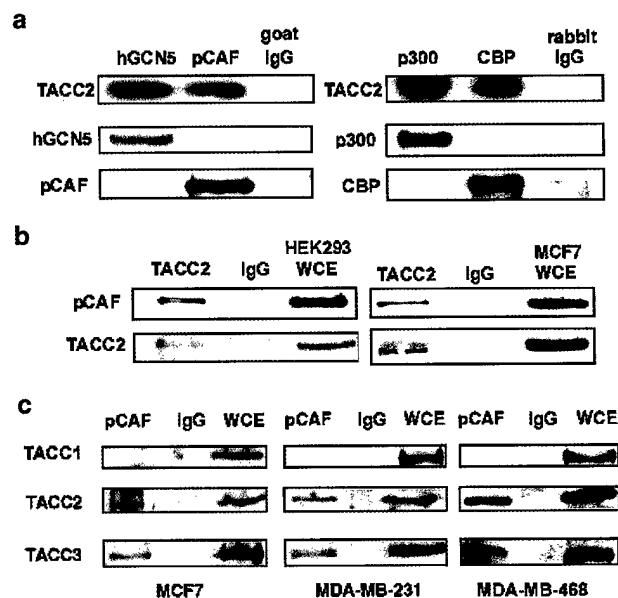
Figure 3 TACC proteins interactions with HATs in cells. (a) HEK293 lysates were immunoprecipitated with goat antibodies to native hGCN5L2, and pCAF, or rabbit polyclonal antibodies to CBP, and p300, or normal species specific IgG, and filters incubated with α-TACC2 antibody. TACC2s is detected in the immunoprecipitates (IPs) of the four HATs, but not in control IgG IPs. (b) pCAF is specifically immunoprecipitated from HEK293 and MCF7 cell lysates by a commercial antibody raised to TACC2. (c) pCAF interacts with TACC2s and TACC3 in breast cancer cells. Cells were immunoprecipitated with mouse α-pCAF or mouse IgG and immunoblotted with α-TACC1, α-TACC2 or α-TACC3 antibody as described in Lauffart et al. (2002). The α-pCAF antibody specifically immunoprecipitates both the native TACC2s and TACC3 protein in MCF7, MDA-MB-468 and MBA-MB-231, but fails to immunoprecipitate TACC1. WCE: whole-cell extract. Commercial antibodies were obtained from the following companies, and used according to the manufacturers' instructions: anti-TACC2 rabbit polyclonal IgG #07-228 (Upstate Biotechnology, Lake Placid NY, USA), anti-hGCN5 (#sc-6303), anti-pCAF (#sc-6300, sc-13124), anti-p300 (#sc-584) and anti-CBP (#sc-369) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Normal IgG and anti-goat-HRP, anti-rabbit-HRP and anti-mouse-HRP conjugates were also obtained from Santa Cruz Biotechnology. The culture of cell lines, preparation of cell lysates, immunoprecipitation and Western blot analysis were performed as described in Lauffart et al. (2002)

Figure 2 In vitro interaction between hGCN5L2 and the TACC proteins. Top panel: Autoradiograph of 10% SDS polyacrylamide gel with in vitro translated hGCN5L2 construct pulled down with GST-TACC1 aa596-ter (T1), GST-TACC2s (T2) aa2-ter, GST-TACC3 aa116-ter (T3) or GST (G). I: 5% input of in vitro translated hGCN5L2 construct. The bottom two panels represent Coomassie blue stained gels of a pull-down experiment showing loading of GST-TACC proteins and GST. Assays were performed as described in Figure 1

expressed in breast cancer cell lines. All of the MDA-MB-231, MDA-MB-468 and MCF7 cell lines expressing TACC2s (Lauffart et al., 2003) also express pCAF (Figure 3c). However, since these cell lines do not express significant levels of hGCN5L2 (data not shown), our coimmunoprecipation analysis was carried out exclusively using an antibody specific for native pCAF. Consistent with our prediction, both native TACC2s and TACC3 were found in the complex containing endogenous pCAF (Figure 3c). However, we were unable to detect any interaction between TACC1 and pCAF in any of these cell lines (Figure 3c), or the immortalized, but not transformed MCF10A breast epithelial cell line (data not shown). Thus, it remains uncertain whether in breast epithelial cells, TACC1 is a preferred binding partner of this HAT.

As stated above, and shown in Figure 4, TACC2 is distributed throughout the cytoplasm and the nucleus of cells in culture. To further confirm that the pCAF and TACC2 proteins could physically interact in the intact cell, we next determined whether pCAF colocalized with endogenous TACC2 in the breast cancer cell line MCF7. Indirect immunofluorescence microscopy demonstrated that native TACC2 is found in both cytoplasmic and nuclear compartments of interphase MCF7 cells (Figure 4b). This distribution has been previously observed for TACC2 in other human cell lines (Chen et al., 2000; Gergely et al., 2000). In these cells, endogenous pCAF is diffusely distributed throughout the nucleus, but is absent from the nucleoli (Figure 4b). Superimposing the images for the two antibodies reveals that both proteins colocalize throughout the nucleus, but are not found in discrete subnuclear structures. This indicates that both the TACC proteins and the HATs can be physically located and potentially interact in the same subcellular compartment.

As presented above, we have demonstrated that the TACC proteins have the ability to bind in vitro and in cellular systems to the members of the HAT family of transcriptional coactivators. In humans, hGCN5L2 copurifies with the SAGA-related transcriptional adaptor complexes STAGA and TFTC (Martinez et al., 1998). The homologous protein, pCAF, also coimmunoprecipitates with factors known to regulate the transcriptional activity of promoters (Ogryzko et al., 1998). In these complexes, the HAT activity of hGCN5L2/pCAF is required for the acetylation of the N-terminal tails of core histones and/or transcription factors, which facilitates the binding of the general transcription machinery, thereby promoting transcription. Thus, the direct interactions of members of the TACC family with the transcription factors ARNT1 (Sadek et al., 2000) and STAT5 (Piekorz et al., 2002), the oncogene GAS41 (Lauffart et al., 2002, 2003), the SWI/SNF core component INI1 (Lauffart et al., 2003), and now the HAT proteins, further support the notion that the TACC family may represent a novel family of transcriptional accessory or regulatory proteins. Indeed, in vivo, immunohistochemical analysis of cross sections of normal human and mouse tissues clearly show that the major subcellular site of one TACC protein,
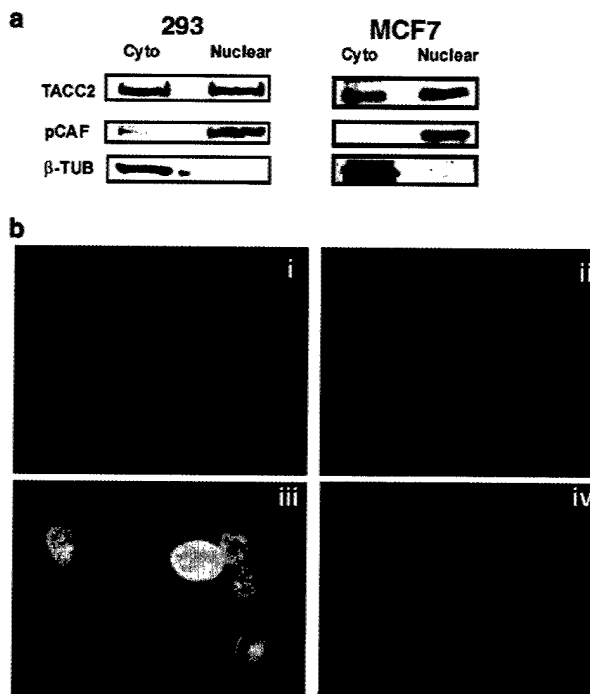
Figure 4 Colocalization of TACC2s and pCAF endogenous proteins in the nucleus of interphase cells. (a) TACC2 is distributed in both nuclear and cytoplasmic (cyto) extracts of HEK293 cells and MCF7 cells. pCAF is predominantly found in the nucleus. The leakage of pCAF protein into the cytoplasmic fraction of HEK293 cells is probably due to nuclear breakdown during extract preparation. Cytoplasmic and nuclear extracts were prepared as described in Schreiber et al. (1989). (b) (i) Indirect immunofluorescence of TACC2 detected with fluorescein isothiocyanate (FITC)-conjugated secondary antibody shows distribution in the cytoplasm and nucleus of MCF7 cells; (ii) nuclear localization of pCAF, detected with Texas red-secondary antibody; and (iii) overlay of the previous images reveals colocalization of both proteins in the nucleus. The green and red colors in the superimposed images result in a yellow color. Nuclei were counterstained with DAPI (panel iv). MCF7 cells were prepared as previously described (Lauffart et al., 2002). Cells on coverslips were incubated with the α-TACC2 polyclonal antibody (Upstate Biotech) together with the α-pCAF mouse monoclonal antibody (Santa Cruz) for 1 h at room temperature. The coverslips were washed with PBS (3 × 5 min.) and incubated with a mixture of FITC-anti-rabbit IgG and Texas red-conjugated anti-mouse IgG for 30 min. Finally, the coverslips were washed with PBS (3 × 5 min), mounted with Vectashield containing DAPI and examined at × 60 magnification (oil). Secondary antibodies were obtained from Jackson Immunolabs

TACC3, is the nucleus indicating that this may be the site of the major function of this protein in interphase cells (Aitola et al., 2003; Sadek et al., 2003).

It is also clear that the function of hGCN5L2 and pCAF is not limited to transcription. hGCN5L2/pCAF also interact with pre-mRNA splicing complexes and DNA-damage repair enzymes, suggesting that acetylation by these HATs is of critical importance to a diverse set of nuclear regulatory events (Martinez et al., 2001). The lack of significant binding of pCAF to TACC1 in breast cancer cell lines may in part be due to a more prominent role of TACC1 in post-transcriptional events, through its interaction with the RNA processing and

transport proteins, LSm7 and SmG (Conte et al., 2002; Lauffart et al., 2002), as opposed to transcriptional regulation. Intriguingly, a post-transcriptional role for the Xenopus TACC protein, maskin, has previously been demonstrated (Stebbins-Boaz et al., 1999). This protein is involved in the regulation of the specific polyadenylation-induced translation of maternal mRNA during oocyte maturation (Stebbins-Boaz et al., 1999), suggesting that the vertebrate TACC proteins may be directly involved at multiple steps during the process of gene expression.

In summary, the findings presented here, together with those from previous studies, clearly indicate that TACC proteins are a multifunctional family of proteins that not only play a role in the functional aspects of mitotic spindle formation during mitosis but may also perform an assembly or coordination function bringing elements of the chromatin remodeling, transcriptional and post-transcriptional machinery together in the interphase nucleus. Based on the results presented in this manuscript and the growing number of basal transcription components with which the TACC

proteins interact, it is tempting to speculate that the interaction of TACC proteins with the SWI/SNF complex and HATs may in part explain the coactivation of hypoxia-induced transcription that has been observed for murine TACC3 (Sadek et al., 2000). Furthermore, this may suggest that the ability of TACC2s to reduce the malignant properties of some breast carcinomas may in part be due to changes in the activity of TACC2–HAT complexes in the regulation of genes governing cell proliferation and metastasis.

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